ENZYMATIC EVALUATION OF PROTEIN QUALITY IN FEED AND FOODSTUFFS

by

STEPHEN LESLIE GRUBB

B. S., Kansas State University, 1968

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1969

Approved by:

Major Professor

TABLE OF CONTENTS

INTRODUCTION

From a nutritional point of view the quality of a protein depends upon the presence and availability of the amino acids, particularly those amino acids that are synthesized to a limited extent or not at all by most animals. In the past, nutritional quality of a protein has usually been evaluated by one of two biologic methods, the nitrogen balance method of Mitchell (1) or Osborne and Mendel's (2) protein efficiency method. In recent years analytic procedures, such as Oser's (3) essential amino acid index and Block and Mitchell's (4) chemical score, have been devised that are based on determination of the amino acids present in food proteins. These procedures, however, have made no allowance for variations in the digestibility and availability of the amino acids present. It was thus implied that a true assessment of the nutritional value of proteins must rely upon biologic evaluations. Sheffner et al. (5) developed the "pepsin-digest-residue amino acid index" later modified by Akeson and Stahmann (6) which compared the pattern of essential amino acids released by in vitro pepsin and pancreatin digestion with the amino acid pattern in the original protein. The object of this study was to apply Akeson and Stahmann's procedure to evaluate protein quality from different vegetable and animal sources and from milled products. A sensitive method would have great value for estimating the biological protein quality when only limited amounts of protein are available.

REVIEW OF LITERATURE

Biological Methods

The essential nature of the proteins in our food supply has long been recognized. The term protein was coined in 1839 by the Dutch chemist, Mulder, to denote the primary importance of these compounds for the animal organism.

The biological evaluation of proteins may be said to date back to 1872 when C. Boit showed that gelatin was unable to support tissue growth and, to 1879 when Rubner first demonstrated a variation in the efficiency with which nitrogen from different proteins was retained in the body.

During the latter part of the nineteenth century, the determination of protein requirements was one of the major problems engaging the attention of students of nutrition. They soon recognized that not all proteins had equal ability to supply the minimum nitrogen requirements of men and animals.

In 1907, Thomas (7) introduced the concept of "biological value" of proteins and by nitrogen balance experiments on human subjects he demonstrated marked differences in the fraction of nitrogen from various sources that could be retained in the body. Such observations focused attention on the possible role of the individual amino acids in nutrition.

Osborne and Mendel (8) furnished the first clear-cut demonstration that animals fail to grow when amino acids are omitted from the diet. They showed that young rats on a diet containing gliadine as the sole protein would not grow unless the amino acid lysine was added and, that when zein protein of corn was the only nitrogen source, both tryptophan and lysine were required for survival and satisfactory growth. They formulated the concept that the function of proteins in the diet, aside from an energy value, was to furnish the organism with those amino acids which the animal organism was incapable

of synthesizing.

Osborne and Mendel (2) evaluated protein quality by the use of rat growth studies. They set a pattern for testing which, revised and simplified over the years, is still the method of choice. In this test the criterion of response was termed "protein efficiency" and was defined as the weight gained per gram of protein consumed. Essentially, the method uses weanling rats carried through a short growth period. The rats are fed the test product at a specified protein level in an otherwise complete diet. It was thought to determine the optimum of protein not only by the absolute amount furnished, but also by its quality.

Osborne and Mendel (2) proposed that the efficiency of the individual protein in this respect must depend on the minimum quantity of any indispensable amino acid.

Based on the work of Thomas (7) a nitrogen balance method for the determination of protein quality was developed by Mitchell (1), Mitchell and Carman (9, 10), and Mitchell et al. (11) which involved the determination of N in feces and urine when a known quantity of the protein under test was the sole source of ingested N. When allowance was made for the N lost during a period of N starvation, the N retained was expressed as a proportion of the N ingested or absorbed. Collection of feces and urine was an essential part of this method and metabolism cages are necessary equipment. Mitchell (1) introduced the formula by which biological value of a protein could be explained.

$$B.V. = \frac{\text{N intake} - (\text{fecal N} - \text{metabolic N}) - (\text{urinary N} - \text{endogenous N}) \times 100}{\text{N intake} - (\text{fecal N} - \text{metabolic N})}$$

This equation expressed the efficiency of the absorbed protein in supplying the amino acids needed for the synthesis of body protein.

Fixsen (12) used carcass analysis to evaluate proteins. It involved estimation of the total N content of the carcass of test animals.

Kosterlitz and Campbell (13) suggested that the sensitivity with which N content of the liver responds to different dietary proteins could be used as the basis of a method for determining the nutritive value of proteins, and such a procedure was outlined by Henry et al (14). It was based on the fact that for relatively small protein intakes values for mg. of liver N/100g. body weight were greater for high-grade than for low-grade proteins.

The use of urine nitrogen ratios as a means of measuring the biological value of proteins was reported by Murlin et al. (15) and Murlin, Hayes and Johnson (16). The method was based on the concept that since creatinine excretion is constant in quantity regardless of diet, and more N is excreted from poor quality than from good quality proteins the ratio of creatinine N to total N in the urine would vary directly with the biological value of the protein being eaten.

A digestive quotient for different organic foods was determined by Manbold (17) who used both a prefeeding and a main feeding period of rats. The prefeeding served the purpose of freeing the digestive tract from all the food previously in it. The digestive quotient was determined during the main period. The food and the stool were analyzed for content of a) ash, b) organic substances, c) raw proteins, d) raw fat, e) raw fiber, and f) N-free extracts. The digestive quotient expressed the percentage of the food retained in the bodies of the animals.

Lassalle et al. (18) found a linear relation between the S : N ratio of the diet and urine in rats fed egg, peanut, and wheat as protein sources.

Equations were derived to predict biological value of protein from these ratios.

A recently developed index of protein evaluation by Goyco and Asenjo (19) was called the lactation value. It depended upon three measurements: body weight change occurring in the lactating mother, gain in body weight of the litter during the first 14 days after birth and the amount of protein consumed by the adult female during this period. The algebraic addition of the body weight changes of mother and litter expressed per gram of protein consumed yielded an index called the lactation value.

Henry and Kon (20) studied the effect of protein intake and age of rats on the biological value of proteins. Using adult rats, they found a higher biological value for casein at 4% rather than at 8% protein levels. Adult rats also gave higher values at both 4% and 8% levels than young rats. Results indicated that sulfur amino acid requirements of the rat decreases with age and that age of an animal should be a consideration when determining the nutritive value of a protein.

Chapman et al. (21) reported protein efficiency ratios, although influenced by the age of rat and subject to certain inherent criticisms, to be a simpler method for evaluating protein quality than determination of net protein quality, the determination of net protein retention or net protein utilization, and equally sensitive.

Protein efficiency ratio determinations carried out by Middleton et al. (22) on a 10% protein diet furnished a valid estimate of the nutritive value of protein and was reported to have several practical advantages.

In conclusion, a number of biological techniques have been used to evaluate proteins but no single procedure for determining protein efficiency of biological value has emerged which has been best for all purposes. A method

may provide more reliable and specific basic scientific information yet be less suitable for the solution of practical animal feeding problems.

In this study he stated that the critical investigator can find a method useful for his purpose which will yield reliable results if they are properly interpreted. However, biological methods require at least enough of the protein source to conduct feeding trials and therefore processed protein sources were very rarely used and processing procedures could not be readily evaluated.

Classification of Amino Acids

When Thomas (7) first used the term biological value he probably had amino acid make-up in mind. It is interesting to note that Thomas developed a concept and a method for its measurement prior to the work of Osborne and Mendel (2) which inaugurated the modern studies of protein quality. Osborne and Mendel noted that tryptophan and lysine are utilized by the animal organism for specific physiological activities and that the relative values of the different proteins in nutrition were based upon their content of those special amino acids which could not be synthesized in the animal body that were indispensable for certain distinct processes.

Rose (23) began his experiments by replacing the proteins in the rat diet with mixtures of highly purified amino acids. Ten amino acids were proven to be indispensable in the diet of rats, the interrelationships between methionine and cystine and between phenylalanine and tyrosine were established; and it was demonstrated that an amino acid mixture of suitable composition could serve effectively as a source of dietary nitrogen.

He established lysine, tryptophan, histidine, phenylalanine, leucine, isoleucine, threonine, methionine, and valine as the amino acids essential

for rat growth. He noted that arginine was essential although it could be synthesized by the animal organism. This rate was not at a sufficiently rapid rate to meet the demands for normal growth. Cystine stimulated growth only when methionine was fed in suboptimal quantities. This was later reaffirmed by Rose (24).

Block and Bolling (25) classified arginine, glycine, cystine, and tyrosine as semi-indispensable amino acids; the first two because they could be synthesized by the animal (or avian) body, but not at rates adequate for maximum growth, and the last two because they are indispensable in food supplies containing less than certain minimal amounts of methionine and phenylalanine, respectively.

Mitchell and Carman (10) found that proteins of whole egg had an amino acid composition that was highly digestible and almost perfectly utilizable in rodent metabolism. This was confirmed by Sommer (26) for both growing and mature rats. Sommer (27) and Sommer et al. (28) found that for the adult human subject whole egg proteins seemed better utilized than whole milk proteins.

The classification of the amino acids was an important advance in the nutritive evaluation of proteins by chemical analysis since attention could now be centered on the essential amino acids. But even so, a precise evaluation of proteins by such means was not at hand to the early workers. Proteins could be compared by graphing their amino acid "contours," or spectra, but 1% of one amino acid in a protein obviously could possess a different nutritive significance than 1% of another. Missing was a yardstick of comparison represented by the amino acid content of a protein or protein mixture which considered availability in digestion and metabolism. It was later found that whole egg provided this yardstick.

Chemical Methods

The relationship of the amino acids to the protein nutritive value for the growing rat was indicated by Block and Mitchell (4). This relationship was shown by computing the percentage deviation of the contents of each essential amino acid, expressed per 16 gm. of nitrogen, from the corresponding contents of a protein that was almost completely digestible by the rat and utilizable in adolescent metabolism. Whole egg was the standard protein used.

From such computations, the essential amino acid limiting the nutritive efficiency of the protein was revealed as that one whose percent deficit from the standard protein (whole egg) was the greatest, due consideration being given to the relationship between cystine and methionine in anabolism. The limiting amino acids thus indicated agreed with those determined in feeding experiments.

They ranked the proteins of foods in order of decreasing nutritive efficiency on the basis of increasing percent deficits of respective limiting essential amino acids. These values were highly correlated (N=0.86) with the corresponding biological values determined by the nitrogen metabolism method.

Oser (3) proposed a somewhat similar chemical method of scoring the nutritive value of proteins by computation of an "essential amino acid index" (EAA index), using egg protein as a standard and computing "egg ratios." The egg ratio was defined as the percentage of each amino acid in the test protein when compared to egg protein. Percentages over 100 were considered as 100 and 0 percentage was given a value of one. The EAA index therefore was the geometric mean of the egg ratios.

Kofranyi (29) determined the biological value of proteins based upon the content of lysine, methionine, tryptophan, and threonine. Lysine was

determined on protein hydrolyzates and the other amino acids were determined biologically.

Lindner (30) separated the amino acids of a protein hydrolyzate by paper chromatography and amounts of amino acids so obtained were determined polarographically through copper complexes and then compared to a standard for determination of biological values.

The availability of amino acids in feeds was studied by Grau and Carroll (31). Their method depended in part on obtaining essentially normal growth when the test protein and a complete amino acid mixture was fed and on a knowledge of the approximate requirements for each essential amino acid. The growth data from these diets, when plotted on a growth response curve provided an estimate of the availability of a specific amino acid.

Rao et al. (32) used the pattern of essential amino acid requirements of the growing rat as a reference protein for estimating the nutritive value of various proteins. He proposed a requirement index based on a chemical estimate of the nutritive value of proteins. Its correlation with biological value was reported as highly significant.

Chemical methods have attempted to show this relationship, however, little or no correlation exists between the chemical ratings of the proteins and their digestibility by the growing rat as reported by Mitchell and Block (33). Mitchell (34) stated that chemical evaluations of proteins by amino acid analysis gives a first approximation of their value in metabolism, but not in digestion. He suggested that it will aid in explaining differences in the metabolic utilization of proteins, and in attaining the most effective supplementation among different protein foods. However, biologic evaluations of proteins and protein mixtures are still the court of last resort.

Many factors are involved in the utilization of dietary proteins that

are unrelated to the chemical availability of amino acids for anabolic purposes. The following factors may be enumerated: differential rates of enzymatic liberation of the amino acids in digestion, differential availability of amino acids in digestion, the time relationships in the ingestion of different proteins.

In assessing the nutritive value of the proteins of heated foods, or foods stored over long periods of time, there are indications that the biologic value, or the digestibility, of the proteins are impaired before the amino acids themselves undergo chemical changes or destruction.

Tarjan (35) criticized the use of the term biological value on the basis of amino acid composition. He pointed out there are discrepancies between chemical composition and dietary values of protein sources. Moreover, biological values at different ages of the organism, such as during youth or in old age as applied to protein sources, may be different. It may be more correct to investigate growth, hemopoisis or the function of the central nervous system, and to decide whether the value of the protein source as found with the current methods of determination is favorable for growth, regeneration or other life functions.

Friedman (36) in a critical analysis of the problem stated that predictions of protein quality cannot be made with complete assurance because the amino acid pattern revealed by analysis may be much altered and distorted by such factors as digestibility, the presence of anti-tryptic substances, processing conditions which result in new chemical linkages not susceptible to enzymatic digestion, etc. The disturbing effect of these factors is illustrated in such foods as legumes in which the availability of methionine varies with the variety; soybean protein, in which proper cooking improves digestion and nutritive value, or overheating reduces the nutritive value

either by destroying essential amino acids or binding them in new linkages so they are not utilizable; corn in which only 25% of the isoleucine may be available; and protein hydrolysates and glucose mixtures that have been incubated for long periods or have been overheated, in which the resulting failure of growth-supporting ability was not predicted by amino acid analyses.

Berg and Rose (37), Geiger (38), and Henry and Kon (39) questioned whether a method which depended in its entirety upon the total amino acid composition could predict precisely the biological value of proteins since many other factors affect the utilization of dietary protein. One of these factors was related to the observation that delayed supplementation of a deficient protein with lacking amino acids was ineffective in correcting deficiencies.

Bacteriological Methods

The use of Tetrahymena geleii H, a protozoan, to estimate biological value of proteins was suggested by Dunn et al. (40). The growth response of the micro-organism to various proteins tested was measured by titrating the acid produced in experimental cultures.

Fernell and Rosen (41) gave details of an assay method using tetrahymena pyriformis under highly aerobic conditions. Growth in relationship to ammonia-N production was taken as an index of the efficiency of protein utilization.

Rogers et al. (42) devised a simplified chemical score using bacteriological methods. Based on the determination of lysine, methionine, or methionine and cystine, a simplified chemical score was developed and compared with protein-efficiency-ratios determined using the same samples. Each food was assigned to either of two categories: a) foods apparently deficient in lysine or b) in methionine (cystine). There was a high correlation between lysine concentration and the protein-efficiency-ratio. Since the regression

lines for the two groups were different a factor was added to the methionine (cystine) values to simplify the relationship. This simplified method was rapid, yielded reproducible results, and correlated with animal assays. It was proposed as a rapid screening procedure for evaluation of protein in food and was not intended to replace the rat biological assay method.

Ford (43) developed a method in which S. faecalis va zynogenes was used to estimate protein quality. Biological values on several meat meals correlated closely with available lysine content. None of the meals was found to be deficient in lysine. Differences in nutritional value seemed to reflect differences in availability rather than in total amounts of several or all amino acids.

Enzymatic Methods

Melnick et al.(44) tried to demonstrate why a given protein may show differences in biological value after heat treatment even though its amino acid composition and degree of digestibility may remain unchanged by supplementing the bio-assay technic with an in vitro digestibility procedure. The in vitro method involved periodic measurements of the degree of hydrolysis of the protein by a modified formal titration procedure.

This indicated that rate of release of individual amino acids during enzymic digestion could readily account for differences in the biological value of proteins.

Riesen et al.(45) proposed that in addition to total amino acid composition, rate of release of amino acids from protein by pancreatic digestion was also an important factor in the nutritional quality of a protein. This concept was utilized by Horn et al.(46) to evaluate the nutritional quality of food proteins by measuring microbiologically the individual amino acids

made available by pepsin, trypsin, and hog mucosa. This method was correlated with the biological value of cottonseed subjected to various degrees of processing.

Dunn, et al. (40) using streptococcus fecalis, and using the proteolytic protozoan, Tetrahymena geleii, developed procedures to estimate the biological value of proteins. They used growth responses of the organisms to a pancreatic digest of the test protein. However, the values obtained with these methods did not correlate well with the biological value of proteins as determined by rat assay. This concept was also utilized by Sheffner et al. (5) in determining the relationship between the biological value of food proteins and the patterns of amino acids released by digestive enzymes, particularly pepsin. He developed an in vitro procedure which accurately estimated the nutritional value of proteins. The pattern of amino acids released in vitro by pepsin revealed differences between proteins which were not apparent from total essential amino acid content. His amino acid index described the physiological availability of amino acids during digestion. The index combined the pattern of essential amino acids released in vitro by pepsin digestion with the amino acid pattern of the remainder of the protein to produce an integrated index. This index was closely correlated with net protein utilization. Division of the index by the digestibility coefficient of the respective proteins yielded values which predicted the biological values of the proteins studied.

Teeri et al. (47) devised a relatively simple and rapid method for the comparative nutritional evaluation of proteins by using the enzymes normally employed in animal digestion and thus measured availability as well as the value of the available protein. He measured the acid produced by Streptococcus faecalis in a medium having amino acids supplied by protein digests.

Chromatographic analysis of the protein digests served to indicate the extent of the hydrolytic process and also showed which amino acids were lacking, or present, in limited amounts in the poor quality proteins.

Gehrt et al. (48) modified the pepsin digestibility method by determining the percentage of indigestible protein in the total sample and proposed that identity and quantity of indigestible residue be determined. Details of an accelerated pepsin digestion method adapted for digestibility of proteins in feed was given by Elmslie (49). The results compared favorably with the method of Sheffner except on a feather meal. A pepsin digestibility method for animal protein feeds has been described in the Association of Official Agricultural Chemists (50). In it a defatted sample is digested 16 hours with warm acid solution of pepsin under constant agitation. Insoluble residue is centrifuged, dried, and weighed, examined microscopically, and analyzed for protein or filtered, washed, and analyzed for protein. The method is suggested for meat scrap, meat and bone scrap, digester tankage, fish meal, whale meal, blood meal, hydrolyzed feather meal, and poultry by-products meal.

Anwar (51) used in vitro digestion of proteins as a grading test for the nutritive value of protein concentrates of both plant and animal origin. Gross protein value, carried out by the simplified technique, was taken as a reference of nutritive value. In general, the method could be applied with a fair degree of accuracy to cottonseed, peanut and meat meals. The response of fish meals to pancreatin digestion did not reveal differences that would account for differences in nutritive value.

Tilley and Terry (52) described a simple laboratory method for the determination of protein quality of forage crops. Steps involved included digestion for 48 hours with acid pepsin followed by weighing the residue. The first stage

was anerobic with gas furnished by the digestion mixture. Reproductibility was good and with sheep the correlation between *in vivo* and *in vitro* digestibility was high.

Akeson and Stahmann (6) investigated a pepsin-pancreatin digest index for estimation of protein quality. The index was calculated from the amino acids released by *in vitro* digestion with pepsin followed by pancreatin digestion. Whole egg was used as a standard. The pepsin-pancreatin digest index values showed better correlation with biological values for the growing rat than did essential amino acid index which tended to underestimate the biological values.

Akeson and Stahmann (53) estimated the nutritive value of leaf protein concentrate by *in vitro* enzymatic digestion. Digestibility was estimated from the total amount of amino acids released by pepsin followed by pancreatin hydrolysis. The biological values of the proteins were estimated from the pepsin-pancreatin digest index, which was based on release of eight essential amino acids. An excellent correlation was observed between the index for reference proteins and their biological values in the literature.

MATERIALS AND METHODS

Part I

Protein digests were prepared by incubating 100 mg. of test protein with 1.5 mg. of pepsin and 15 mg. of 0.1 N HCl at 37° C for 3 hours. After 3 hours, the digestion mixtures were neutralized with 7.5 ml. of 0.2 N NaOH and incubated for an additional 24 hours at 37° C using 4 mg. of pancreatin in 7.5 ml. of pH 8.0 phosphate buffer which contained 50 ppm. merthiolate. Enzyme blanks were prepared by incubation under the described conditions with the protein sample omitted. Fifty parts per million merthiolate added to the digestion mixture to prevent growth of microorganisms did not interfere with the digestion and subsequent analysis after 24 hours of digestion. Ten milliliters of digestion mixture were added to 50 ml. of one percent picric acid solution to precipitate the undigested proteins and peptides. This mixture was frozen and stored for 24 hours and then filtered through a Whatman No. 4 filter.

Fifty milliliters of the filtrate were passed through a 12 x 50 mm. column of Dowex 2 x 8 anion exchange resin in chloride form to remove the picric acid. The column was rinsed three times with 5 ml. portions of 0.02 N HCl. The amino acid digest mixture was then evaporated to dryness using vacuum distillation equipment. The dried samples were dissolved and diluted to 10 ml. with pH 2.2 citrate buffer. Amino acid analysis of the samples was conducted using ion exchange chromatography as described by Moore et al. (54), with a Beckman model 120 B amino acid autoanalyzer. Basic amino acids were separated on a 10 cm. column using pH 5.28 buffer and acidic and neutral amino acids were separated on a 159 cm. column using pH 3.25 buffer followed by pH 4.25 buffer after 1 hour 40 minutes from zero time.

The total amino acid content of the samples was estimated on acid hydrolysates.

Samples were hydrolyzed using 2 ml of 6 N HCl, sealed under a vacuum of 27.4 inches of Hg, for 22 hours at 110° C. After filtration and evaporation to dryness 3 times with vacuum distillation, the samples were dissolved in pH 2.2 citrate buffer and diluted according to the protein content of the sample. Amino acid analysis of the digests was conducted using the method described for pepsin-pancreatin digests.

The pepsin-pancreatin digest index used to estimate biological values was calculated using gm of amino acid per 100 gm Kjeldhal protein.

Evaluations included seven food proteins selected to cover a range of protein quality. Commercial preparations tested were whole egg, casein, full fat soybean flour, 50% soybean meal protein, wheat flour, brewers' dried yeast, dried whole whey, and corn. The estimated biological values of the seven reference proteins were compared to literature values and a regression line was derived to predict biological values of proteins not found in literature.

Part II

Digests of 10 protein samples from milled fractions of sorghum grain were prepared as described in Part I. Their biological values were estimated from the regression equation derived from Part I. Feeding tests were conducted with the same protein sources and protein efficiency ratios (PER) were determined after four weeks. The estimated biological values and calculated PER's from the feeding trials were compared and evaluated. A regression equation based on the pepsin-pancreatin digest was calculated which would estimate PER's for the protein. Biological values and PER's were estimated for protein sources when these values were not available in the literature.

RESULTS AND DISCUSSION

The amino acids used in all calculations included lysine, phenylalanine plus tyrosine, methionine, threonine, valine, isoleucine, leucine, and histidine. These were the amino acids classified by Rose (23) as essential for the growing rat. Tryptophan, partially destroyed during the picric acid procedure, was not determined in the enzyme hydrolysates. It was also destroyed by acid hydrolysis and was therefore not used in the calculations. Rose (24) showed the minimal level of tryptophan required for rat growth was lower (0.2%) than required levels of other essential amino acids. Therefore, good estimations of the biological value should be possible for the majority of proteins without including tryptophan. This eliminated the need for a separate analysis for tryptophan.

Arginine which has been classified as a semi-indispensable amino acid for the rat and therefore required in only small amounts (24) was not included in the calculations of protein quality.

Figure 1 shows typical chromatograms for acid hydrolysates and pepsin-pancreatin digests. The amino acids used in all calculations are identified and labeled with the exception of tryptophan. Although tryptophan is partially destroyed in pepsin-pancreatin digest procedures during addition of picric acid a small peak did appear on the chromatograms and should be present to insure a successful pepsin-pancreatin digest. It was found that if this peak was not present lower values for all the amino acids and especially lysine and histidine were found. When the peak did not appear the pepsin-pancreatin digest was repeated. If tryptophan again did not appear it was assumed that the sample was devoid of tryptophan and the biological value was estimated for the protein source.

As shown in Fig. 1, excellent resolution of the amino acids was obtained with the pepsin-pancreatin digest with the exception of cystine which did not appear

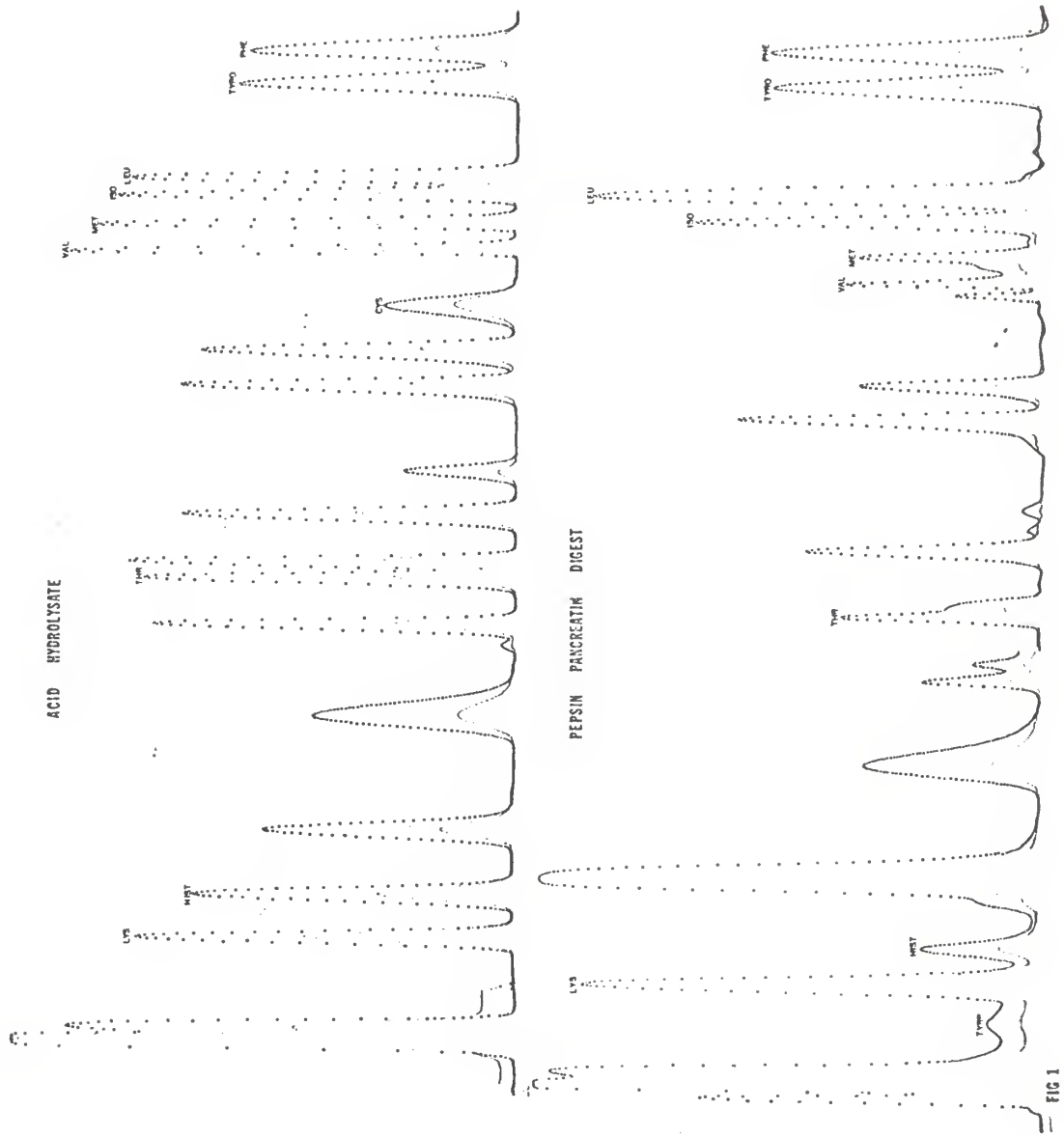

Fig. 1. Patterns of Amino Acids Released by Acid and Enzymatic Hydrolysis

in the chromatograms shown. This was observed in most samples tested but cystine when present appeared in the same place as cystine of the acid hydrolysate. It is also noted that threonine did not separate completely from serine. This was observed in all foodstuffs tested. Interference can be shown in the area where the pattern of the amino acids do not reach the base line. This interference is thought to be from peptides too small to be precipitated by the picric acid (6). In those areas showing peptide interference a slanted line was drawn and served as a base line for integrating the amino acid peaks.

Following the report of Mitchell and Block (33) whole egg protein was used as the reference protein for the calculations of biological value and protein utilization. Its value was set at 100 since it has the highest biological value, as determined by rat studies, of the proteins tested.

The pepsin-pancreatin digest index was calculated in essentially the same manner as the "pepsin-digest-residue amino acid index" described by Sheffner et al. (5). The concentration of each amino acid (g. of amino acids per 100 g. of Kjeldhal protein) found in the pepsin-pancreatin digest was subtracted from the concentration of that amino acid in the total hydrolysate to give the residue fraction. Each amino acid was then calculated as the percentage of the sum of the 10 amino acids for the protein in the pepsin digest and residue fractions, respectively. An example using whole egg and casein is shown in Tables I and II. The ratio of the percentage of each amino acid in the pepsin digest of casein to the percentage of that amino acid in the pepsin digest of whole egg gives the "egg ratio." The geometric mean of the adjusted egg ratios was then computed logarithmically by averaging the logarithms of the egg ratios, and obtaining the antilogarithm. A similar calculation was made for the residue fraction. Egg ratios of less than one were considered as one in order to avoid negative logarithms. This is illustrated in Table II.

TABLE I. EXAMPLE OF CALCULATIONS TO ESTIMATE BIOLOGICAL VALUE OF CASEIN FROM AMINO ACIDS RELEASED BY ACID AND ENZYMATIC HYDROLYSATES

Whole Egg

Amino Acid	Total Hydrolysate	Digest	Residue
Lysine	8.290[a]	1.303[a,b]	6.987[a]
Histidine	2.794	0.197	2.597
Threonine	4.962	0.714	4.248
Cystine	5.115	0.609	4.506
Methionine	3.616	0.946	2.670
Valine	6.965	1.386	5.579
Isoleucine	6.085	0.946	5.139
Leucine	9.774	3.330	6.436
Tyrosine	4.615	1.589	3.026
Phenylalanine	6.241	1.786	4.455
Total	58.457	12.773	45.684

[a]Grams of amino acid per 100 grams Kjeldhal protein

[b]Corrected from blank

TABLE I CONTINUED

Casein

Amino Acid	Total Hydrolysate	Digest	Residue
Lysine	7.414[a]	1.613[a,b]	5.801[a]
Histidine	2.653	0.179	2.474
Threonine	3.893	0.564	3.329
Cystine	0.478	0.000	0.478
Methionine	2.426	0.532	1.894
Valine	5.776	0.842	4.934
Isoleucine	4.785	0.580	4.205
Leucine	8.652	2.222	6.430
Tyrosine	5.226	1.287	3.939
Phenylalanine	4.657	1.212	3.445
Total		9.031	36.929

[a]Grams of amino acid per 100 grams Kjeldhal protein

[b]Corrected from blank

TABLE II. EXAMPLE OF CALCULATIONS TO ESTIMATE BIOLOGICAL VALUE OF CASEIN FROM AMINO ACIDS RELEASED BY ACID AND ENZYMATIC HYDROLYSATES (DIGEST)

Amino Acid	Casein Digest Ratio	Egg Digest Ratio	Casein/ Egg Ratio Ratio	Logarithmic Value
Lysine	17.9	10.2	100.0	2.0000
Histidine	2.0	1.6	100.0	2.0000
Threonine	6.2	5.6	100.0	2.0000
Cystine	0.0	4.8	48.4	1.6848
Methionine	5.9	7.4		
Valine	9.3	10.8	68.1	1.8331
Isoleucine	6.4	7.4	86.5	1.9370
Leucine	23.6	26.1	90.4	1.9562
Tyrosine	14.3	12.5	98.0	1.9912
Phenylalanine	13.4	14.0		
Total				15.4023
Average				1.9253
Anti-Log				84.2

TABLE II CONTINUED

Amino Acid	Casein Digest Ratio	Egg Digest Ratio	Casein/ Egg Ratio Ratio	Logarithmic Value
Lysine	15.7	15.3	100.0	2.0000
Histidine	6.7	5.7	100.0	2.0000
Threonine	9.0	9.3	96.7	1.9854
Cystine	1.3	9.9	40.7	1.6096
Methionine	5.1	5.8		
Valine	13.4	12.2	100.0	2.0000
Isoleucine	11.4	11.2	100.0	2.0000
Leucine	17.4	14.2	100.0	2.0000
Tyrosine	10.7	6.6	96.9	1.9863
Phenylalanine	9.3	9.6		
Total				15.5813
Average				1.9477
Anti-Log				88.7

Correcting for Degree of Proteolysis:

Digest $84.2 \times \frac{9.031}{12.773} = 59.5$

Residue $88.7 \times \frac{36.929}{45.684} = 71.7$

In computing the egg ratios, percentage concentrations of amino acids in excess of those present in the standard protein were disregarded. Methionine and cystine were considered as a unit, as were phenylalanine plus tyrosine. If the essential precursor amino acid of the pair was present in excess of that in egg, the excess was used to make up a deficiency of the nonessential amino acid, but the reverse was not done.

The geometric means of the fractions were each multiplied by a factor to correct for the degree of proteolysis of the test protein relative to that for the standard egg protein. The factor for the pepsin-pancreatin digest was obtained by summing the concentrations of the 10 individual amino acids in the pepsin digest of the test protein and dividing the total by the sum of the concentrations obtained for the standard egg. Multiplying the geometric mean of the two fractions by their respective proteolysis factors yielded the corrected geometric mean as shown in Table II.

To obtain an amino acid index for the whole protein the corrected geometric mean of the pepsin digest fraction and the residue fraction was weighed in accordance with the percentage each represented of the total standard egg protein. The corrected geometric means were weighed and averaged geometrically to obtain the pepsin-pancreatin index for the whole protein. The index was divided by the in-vitro protein digestibility value to obtain an estimated biological value for the protein as follows:

Correction for Standard Egg Protein

Log. 59.5 = 1.7745

Log. 71.7 = 1.8555

$$(1.7745 \times \frac{12.773}{58.457} + 1.8555 \times \frac{45.684}{58.457}) = \text{Index Value}$$

0.3886 + 1.4491 = 1.8377

Antilog 1.8377 = 68.8

68.8/Digestibility value = Estimated biological value

68.8/97 = 70.9

Estimated Biological Value of Casein = 71

The estimated biological values calculated from pepsin-pancreatin digests and acid hydrolysates of the reference protein are shown in Table I, Column 2. The biological values for growing rats as reported in the literature for the seven proteins are shown in Column 3. One or more reports of the biological values were found for each protein source and these values were averaged for comparison studies.

The correlation coefficient between the calculated biological values of the reference proteins and literature biological values was calculated to be ($r = 0.991$). Figure 2 represents the relationship between the biological values from the literature and the calculated values. The regression line ($y = 0.17x + 0.54$) shows this relationship, and indicates the calculated values may slightly overestimate the true biological values. The standard error of the estimate is small and not significant at either the 1% or 5% level.

The estimated biological value of protein sources might therefore be predicted from the equation ($y = 0.970x + 0.54$) where y = the predicted value and x = the number obtained from the pepsin-pancreatin digest and acid hydrolysate calculations.

TABLE III. COMPARISON OF BIOLOGICAL VALUES ESTIMATED BY IN VITRO DETERMINATION AND BIOLOGICAL VALUES FROM LITERATURE FOR THE GROWING RAT

Food Protein	Pepsin-Pancreatin Biological Values	Literature Biological Values for Growing Rats	
		Range	Average
Whole Egg	100	100 - 97[1,2,3]	98
Casein	71	68 - 78[2,4]	72
Soybean 44%	66	Raw 57 - 59 Heated 75 - 74[2,4]	66
Soybean 50%	68		
Yeast (Brewers' Dried 47% Protein)	67	63 - 69[2,3]	66
Wheat Flour (Hard Red Winter 12%)	53	52[2,3]	52
Corn (#2 Dent 10%)	60	60[5]	60
Whey (Whole Dried 13.5%)	57	60 - 63[2]	61

[1]Sommer (26)

[2]Mitchell and Block (33)

[3]Mitchell and Carman (9, 10)

[4]Riesen (45)

[5]Fixsen (12)

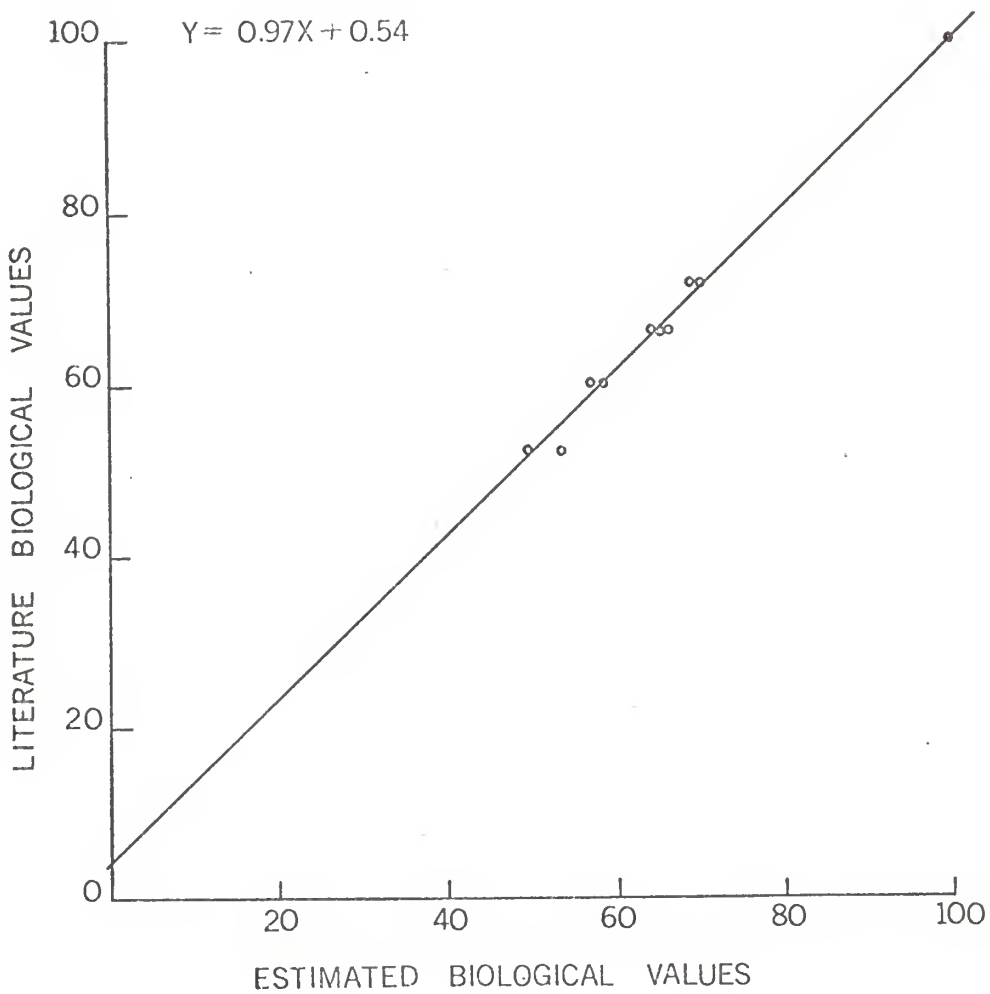

Fig. 2. Relationship Between Values Estimated by In Vitro Determination and Biological Values from Literaure for the Growing Rat

TABLE II. COMPARISON OF ESTIMATED BIOLOGICAL VALUES AND PER VALUES CALCULATED FROM TWO RAT STUDIES

Protein Source	Crude Protein	Grams of Gain per Gram of Protein Consumed (PER)	Predicted Biological Value
Casein	87.0	2.50	71
Sorghum Grain Milling Fractions			
A	10.6	2.08	64
B	10.4	1.04	54
C	6.0	.776	52
D	6.5	.580	51
E	5.7	.495	49
F	9.8	.390	46
G	13.8	.273	47
H	14.3	.165	46
I	18.0	.160	43
J	15.0	.120	41

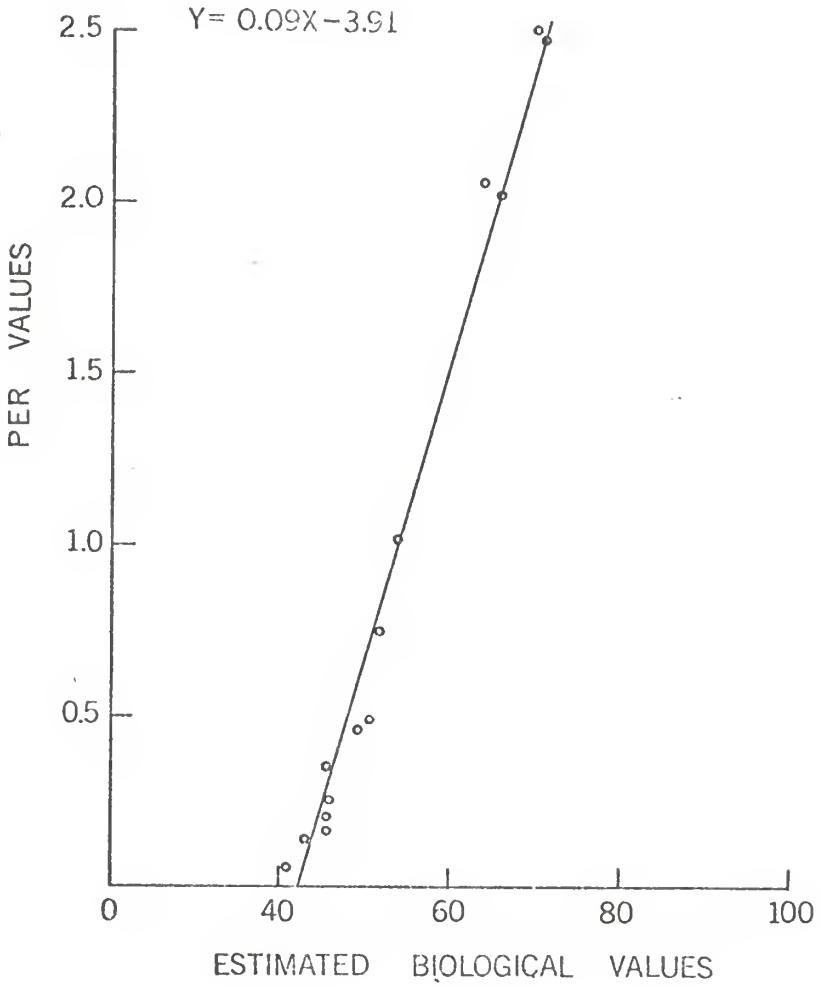

Fig. 3. Relationship Between Estimated Biological Values and PER Values Calculated from Rat Studies

calculated PER values for the protein source based on the rat growth studies and Column 3 shows the predicted biological values of these proteins based on the pepsin-pancreatin digests. A scatter diagram of the data was drawn and the regression line, ($y = 0.09x - 3.91$), derived from the data is illustrated in Fig. 3. The calculated coefficient between the predicted biological values and the PER's resulted in a positive and significant correlation, ($R = 0.982$). The regression standard error of the estimate was small and not significant at the 5% or 1% level. Hence the predicted biological value would be useful in predicting or estimating PER values.

Digests and hydrolysates of fractions of corn tassel and yeast were prepared as in Part I. Biological values and PER's have not been established for these protein sources. A digestibility index was established and biological values were estimated from the regression equation established in Part I. PER values were predicted using the regression equation established in Part II where y = predicted PER value and x = the estimated biological value. The estimated values for the food proteins are shown in Table V.

TABLE V. ESTIMATED BIOLOGICAL AND PER VALUES OF PROTEIN SOURCES NOT REPORTED IN LITERATURE

Protein Source	Predicted Biological Values	Predicted PER
Corn Tassel (25% protein)	42	0.00
Corn Tassel (18% protein)	63	1.85
Yeast (27% Coors Dried Brewers' Grains)	64	1.95

The data presented on these products represents evaluation of protein sources in which the source of protein was too small for feeding trials. Approximately 150 mg. of protein were used and the time involved in the determination was 4 days.

These protein sources may contain factors other than amino acids which add to their nutritive value and therefore all protein foods evaluated by this method should be, in the final analysis, tested with feeding trials because any evaluation based on amino acid analysis does not take into account the contribution to the welfare of the animal of factors other than amino acids.

SUMMARY

The pepsin-pancreatin digestion procedure can be used to screen protein sources due to its ability to estimate closely the biological value. Also the amount needed for this determination is approximately 150 mg. of protein. A third advantage of the determination is total time involved which is approximately 3 - 4 days.

The evaluation test can be used to estimate PER values. This would be especially useful when evaluating processed proteins in which samples too small for rat studies are obtained. The determination can also be useful in evaluating processing procedures.

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Charles W. Deyoe, my major professor, for his guidance and assistance in both the research and the preparation of this manuscript.

To Dr. William J. Hoover, head of the Department of Grain Science and Industry, appreciation is extended for the use of research facilities to conduct this study.

I also wish to thank Dr. Howard L. Mitchell and Dr. Paul E. Sanford for their being on the advisory committee and reviewing the manuscript.

Thanks also to Mr. Floyd Shoup for supplying the rat study data and to Jo Ann Bathurst for her assistance in the preparation of samples. I also appreciate the assistance given by other members of the staff and faculty.

BIBLIOGRAPHY

1. Mitchell, H. H.
A method of determining the biological value of proteins. J. Biol. Chem. 58-873-903 (1924)

2. Osborne, T. B. and L. B. Mendel
A method of expressing numerically the growth-promoting value of proteins. J. Biol. Chem. 37:223-9 (1919)

3. Oser, B. L.
Method for integrating essential amino acid content in the nutritional evaluation of protein. J. Am. Dietet. Assn. 27:396 (1951)

4. Block, R. J. and H. H. Mitchell
The correlation of the amino acid composition of proteins with their nutritive value. Nutrition Abstr. Rev., 16:249 (1946)

5. Sheffner, A. L., G. A. Eckfeldt and H. Spector
The pepsin-digest-residue amino acid index of net protein utilization. J. Nutr. 60:105 (1956)

6. Akeson, W. R. and M. A. Stahmann
A pepsin-pancreatin digest index of protein quality evaluation. J. Nutrition. 83:257 (1964)

7. Thomas, T. R.
Proteins in nutrition and growth. J. Biol. Chem. 2:62-68 (1907)

8. Osborne, T. B. and L. B. Mendel
Amino acids in nutrition and growth. J. Biol. Chem. 17:325-49 (1914)

9. Mitchell, H. H. and G. G. Carman
The biological value for maintenance and growth of the proteins of whole wheat, eggs, and pork. J. Biol. Chem. 60:613-20 (1924)

10. Mitchell, H. H. and G. G. Carman
The biological value of the nitrogen of mixtures of patent white flour and animal foods. J. Biol. Chem. 68:183-215 (1926)

11. Mitchell, H. H., T. S. Hamilton and R. J. Beadles
The importance of commercial processing for the protein value of food products. I. Soybean, coconut and sunflower seed. J. Nutrition. 29:13-25 (1945)

12. Fixsen, Margaret A. Boas
The biological value of protein in nutrition. Nutrition Abstr. Rev. 4:447-59 (1935)

13. Kosterlitz, H. W. and Rosa M. Campbell
Assay of the biological value of a protein by its effect on liver cytoplasm. Nature. 157:628 (1946)

14. Henry, Kathleen M., H. W. Kosterlitz and M. H. Quenouille
A method for determining the nutritive value of a protein by its effect on liver protein. Brit. J. Nutrition. 7:51-67 (1953)

15. Murlin, John R., T. A. Szymanski and Elizabeth C. Nasset
Creatinine nitrogen percentage as a check on the biological values of proteins. J. Nutrition. 36:171-5 (1948)

16. Murlin, John R., A. D. Hayes and Karl Johnson
Correlation between the biological value of proteins and the percentage of creatine in the urine. J. Nutrition. 51:149-61 (1953)

17. Mangold, E.
A method to determine the digestibility of feeds. Arch. Tierernahr. 1:188-91 (1950)

18. Lassalle, Carlos, Maria Angelica Tagle and Gonzalo Donoso
Prediction of the protein value of the diet from the sulfur and nitrogen content of the urine. Nutrition. 3:22-7 (1964)

19. Goyco, Jose A. and Conrodo F. Asenjo
The lactation value, a new index of protein evaluation. J. Nutr. 85(1):52-6 (1965)

20. Henry, Kathleen M. and S. K. Kon
Effect of level of protein intake and of age of rat on the biological value of proteins. Brit. J. Nutr. 11:305-13 (1957)

21. Chapman, D. C., Raul Castillo and J. A. Campbell
Evaluation of protein in foods. I. A method for the determination of protein efficiency ratios. Can. J. Biochem. and Physiol. 37:679-86 (1959)

22. Middleton, E. J., A. B. Morrison, and J. A. Campbell
Evaluation of protein in foods. Can. J. Biochem. and Physiol. 38:865-9 (1960)

23. Rose, W. C.
Nutritive significance of the amino acids and certain related compounds. Science. 86:298 (1937)

24. Rose, W. C.
Nutritive significance of the amino acids and certain related compounds. Physiol. Rev. 18:109 (1938)

25. Block, R. J. and D. Bolling
Nutritional opportunities with amino acids. J. Am. Dietet. Assn. 20:69 (1944)

26. Sommer, E. E.
Biological value of milk and egg protein in young and mature rats. J. Nutr. 16:129 (1938)

27. Sommer, E. E.
Biological value of milk and egg protein in human subjects. J. Nutr. 16:141 (1938)

28. Sommer, E. E., H. B. Pierce and J. R. Murlin
Egg-replacement value of several proteins in human nutrition. J. Nutr. 16:37-56 (1938)

29. Kofranyi, E.
Determination of the biological value of food protein. II. Lysine determination. Z. Physiol. Chem. 305:61-2, 63-9 (1956)

30. Lindner, Kardly
Polarographic evaluation of the biological value of proteins. Acta Chim. Acad. Sci. Hung. 9:353-62 (1956) (English summary)

31. Grau, C. R. and R. W. Carroll
Evaluation of protein quality in processed plant protein foodstuffs. A. M. Alschul Academic Press, New York, p. 153 (1959)

32. Rao, P. B. Rama, H. W. Norton and B. Connor Johnson
The amino acid composition and nutritive value of proteins. V. Amino acid requirements as a pattern for protein evaluation. J. Nutr. 82(1):88-92 (1964)

33. Mitchell, H. H. and R. J. Block
Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Bio. Chem. 163:599 (1946)

34. Mitchell, H. H.
The nutritional evaluation of protein: A half century of progress. Nutritional Reviews. 10:33 (1952)

35. Tarjan, R.
The biological value of food protein. Inds. Agr. et Aliment. 74:357-60 (1957)

36. Friedman, Leo
Evaluation of protein quality. I. The significance of the problem. J. Assoc. Offic. Agr. Chemists. 41:188-91 (1958)

37. Berg, C. P. and W. C. Rose
Tryptophan and growth. I. Growth upon a tryptophan deficient basal diet supplemented at varying intervals by the separate feeding of tryptophan. J. Biol. Chem. 82:479-84 (1929)

38. Geiger, E.
Experiments with delayed supplementation of incomplete amino acid mixtures. J. Nutrition. 34:97-111 (1947)

39. Henry, K. M. and S. K. Kon
The biological value of the proteins of dried skim milk: value as an addition to bread; tests with young and older rats effects of storage. J. Dairy Research. 14:403-13 (1946)

40. Dunn, M. S., L. B. Rockland, D. Anderson and C. L. Williams
Biological value of proteins determined with Tetrahymena geleii H. Proc. Soc. Exp. Biol. Med. 64:377-379 (1947)

41. Fernell, W. R. and G. D. Rosen
Microbiological evaluation of protein quality with tetrahymena pyriformis W. I. Characteristics of growth of the organism and determination of relative nutritive values of intact protein. Brit. J. Nutrition. 10:143-56 (1956)

42. Rogers, C. G., J. M. McLauglan, and D. G. Chapman
Evaluation of protein in foods. IV. A simplified chemical score. Can. J. Biochem. and Physiol. 37:1351-60 (1959)

43. Ford, J. E.
A microbiological method for assessing the nutritional value of proteins. III. Further studies on the measurement of available amino acids. Brit. J. Nutr. 18(3):499-60 (1964)

44. Melnick, D., B. L. Oser and S. Weiss
Rate of enzymic digestion of proteins as a factor in nutrition. Science. 103:326-329 (1946)

45. Riesen, D. R. C., C. A. Elvehjem and W. W. Cravens
Liberation of essential amino acids from raw, properly heated, and overheated soybean-oil meal. j. Biol. Chem. 167:143-50 (1947)

46. Horn, Millard J., Amos E. Bluh, Madelyn Womack and Charles E. F. Gersdorff
Nutritional evaluation of food proteins by measuring availability of amino acids to microorganisms. I. Cottonseed proteins. J. Nutr. 48:231 (1952)

47. Terri, A. El, W. Virchow and M. E. Loughlin
A microbiological method for the nutritional evaluation of proteins. J. Nutr. 59:587-93 (1956)

48. Gehrt, Albert J., M. J. Caldwell and W. P. Elmslie
Modifications of pepsin-digestibility method for animal proteins. J. Assoc. Offic. Agr. Chemists. 40:606-17 (1957)

49. Elmslie, W. P.
Digestibility of proteins. J. Assoc. Offic. Agr. Chemists. 41: 233-40 (1958)

50.
Official methods of analysis of the Association of Official Agricultural Chemists. 9:286 (1960)

51. Anwar, A.
Evaluation of proteins by *in vitro* pancreatin digestion. Poultry Sci. 41:1120-3 (1962)

52. Tilley, J. M. A., and R. A. Terry
A two stage technique for the *in vitro* digestion of forage crops. J. Brit. Grassland Soc. 18(2):104-11 (1963)

53. Akeson, W. R. and M. A. Stahmann
Nutritive value of leaf protein concentrate, and *in vitro* digestion study. Agr. and Food Chemistry. 13(2):145 (1965)

54. Moore, Stanford, Darrell H. Spakman and William H. Stein
Chromatography of amino acids on sulfonated polystyrene resins. An improved system. Analytical Chemistry. 30(7):1185-90 (1958)

ENZYMATIC EVALUATION OF PROTEIN QUALITY IN FEED AND FOODSTUFFS

by

STEPHEN LESLIE GRUBB

B. S., Kansas State University, 1968

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1969

A rapid method to estimate biological value by means of a pepsin-pancreatin digest was developed. The reference proteins used in these studies were casein, soybean, yeast, wheat flour, corn, and whey. Estimated biological values were calculated from eight essential amino acids plus the tyrosine and cystine released by *in vitro* digestion. The method was based on digestion with pepsin followed by pancreatin. The results of the pepsin-pancreatin digest was compared to an acid hydrolysate. The determination required approximately 150 mg of protein and 3 - 4 days total time. Excellent correlations for reference proteins were observed between literature values and pepsin-pancreatin digest values. The estimated values slightly overestimated the values found in the literature with an estimated biological value of 100 for whole egg and values of 71, 66, 68, 67, 53, 60 and 57 for casein, soybean protein (44%), soybean protein (50%), brewers dried yeast, wheat flour, corn and whey.

The biological values of milled grain sorghum were estimated using the described method and compared with PER values determined by rat studies. An excellent positive correlation was observed between the two values. Predicted PER values were made on samples of corn tassel and indicate the methods application to other protein sources.